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Evaluation of Two rK39 Dipstick Tests, Direct Agglutination Test, and Indirect Fluorescent Antibody Test for Diagnosis of Visceral Leishmaniasis in a New Epidemic Site in Highland Ethiopia

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Abstract. We assessed the performance characteristics of two rK39 immunochromatographic tests, a direct agglutination test (DAT), and an indirect immunofluorescent antibody test (IFAT) in the site of a new epidemic of visceral leishmaniasis (VL) in northwestern Ethiopia. The study population was composed of 179 patients with suspected VL and 67 controls. The sensitivities of Kalazar Detect®, DiaMed-IT Leish®, DAT, and IFAT in 35 polymerase chain reaction–confirmed VL cases were 94.3%, 91.4%, 91.4%, and 100%, respectively, and the specificities were 98.5%, 94%, 98.5%, and 98.5%, respectively. In a Bayesian latent class analysis of all 246 specimens, the estimated sensitivities were 90.5%, 89%, 88.8%, and 96% for Kalazar Detect®, DiaMed-IT Leish®, DAT, and IFAT, respectively; DAT showed the highest estimated specificity (97.4%). Both rK39 immunochromatographic tests perform as well as DAT, and are suitable for VL diagnosis in first-level health centers in this area of Ethiopia.

INTRODUCTION

Visceral leishmaniasis (VL) is caused by protozoan parasites of the *Leishmania donovani* complex that invade and multiply primarily in the spleen, liver and bone marrow.[1] The disease is characterized by prolonged fever, weight loss, splenomegaly and pancytopenia, and carries a high risk of mortality in the absence of treatment. Parasite demonstration in tissue smears and/or culture provides definitive diagnosis of VL, but generally has lower sensitivity than serologic methods.[2] Splenic aspirate has the highest sensitivity of available tissue sampling techniques, but carries a risk of serious hemorrhage and requires an experienced microscopist, limiting its utility for peripheral health facilities.[3] Bone marrow and lymph node aspirates are safer, but have substantially lower sensitivity and similar operational constraints.[2] Classically, parasites have been detected in tissue samples by light microscopy of stained slides or culture in a specialized medium. However, microscopy lacks sensitivity, whereas culture requires weeks to yield a final result and is vulnerable to bacterial contamination. In recent years, molecular techniques have been shown to have the highest diagnostic sensitivity in aspirate or tissue samples and to be highly specific in experienced hands. However, use of the polymerase chain reaction (PCR) remains largely restricted to referral hospitals and research centers.[4]

Conventional serologic tests, such as the indirect immunofluorescent antibody test (IFAT), have high sensitivity because of the presence of high levels of IgG against *Leishmania* spp. in the serum of otherwise immunocompetent VL patients.[5,6] Spurred by the need for non-invasive diagnostic testing in the limited resource settings most affected by the disease, several field-applicable serologic tests have been developed since the 1990s.[7] The first test to be developed was the direct agglutination test (DAT); this test can be read visually without a machine reader, but requires trained technicians, specialized reagents and plates, and initially cost $10 or more per test.[8,9] The cost of DAT antigen used at the Addis Zemen Health Center in Ethiopia was approximately $4 per test. Subsequently, several immunochromatographic tests (ICTs) based on the recombinant K39 antigen (rK39), comprising the 39 amino acid repeats of a kinesin-like gene from *Leishmania chagasi,* have been validated in several leishmaniasis-endemic areas.[10] The ICTs require minimal equipment, can be applied in peripheral health posts and community settings, and range in price from $1.50 to $3.00. A meta-analysis of 30 studies of DAT and 13 studies of rK39 ICTs concluded that their overall diagnostic performance was good to excellent, but that rK39 ICTs had lower sensitivity and less consistent performance characteristics in eastern Africa compared with other regions.[11–17] Nevertheless, changes in prototypes and marketed tests over time as well as differences in study protocols complicate comparisons.

This analysis used specimens collected during an investigation in a new VL epidemic site in northwestern Ethiopia.[18] Our major objective was to address a need on the part of the Ethiopian Ministry of Health for local validation of two commercially available VL rapid tests. To address this issue, we evaluated the performance of four serological tests (DiaMed and InBios ICTs, DAT and IFAT) in two analyses: 1) in a group of specimens confirmed positive by *Leishmania*-specific PCR and 2) in a latent class analysis of data from a larger group of specimens from suspected VL cases. In both cases, specificity evaluation was conducted using specimens from healthy controls from the same disease-endemic area.

MATERIALS AND METHODS

Study site. This study was conducted at the AZHC, which is staffed by physicians and other professionals from Médecins

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Sans Frontières (Operational Center Barcelona-Athens). The AZHC is the only health care facility for diagnosis and treatment of VL patients in the *weredas* (districts) of Libo and Fogera in the highlands of the Amhara Region of northwestern Ethiopia. The specimens used in this analysis were collected during the VL epidemic investigation described in previous publications.[18–20] Informed consent was obtained from all the adults who participated in the outbreak investigation. For young children, consent was obtained from parents or guardians. The protocol was reviewed and approved by the Ministry of Health–Ethiopia, the World Health Organization (WHO)–Ethiopia, and Centers for Disease Control and Prevention, and judged to be covered as an outbreak investigation. Data were delinked from personal identifiers prior to this analysis.

Patient recruitment. Patient recruitment was carried out during two periods of time: 1) October 8–22, 2005, during a rapid outbreak assessment by local and national health authorities with technical support from the WHO[18] and 2) February 5–24, 2007, when a team of investigators returned to the Amhara Region to conduct a case–control study to evaluate risk factors for VL in the outbreak.[20] Cases were defined based on the Médecins Sans Frontières VL case definition as modified for a malaria-endemic area, requiring fever for > 2 weeks, splenomegaly and/or abdominal swelling, and/or weight loss; malaria was ruled out based on a rapid antigen detection test.[21] Patients who fulfilled this case definition were eligible to participate as suspected VL patients. We included untreated patients and those being treated with antimonial drugs at the time of recruitment. Three patients with apparent antileishmanial treatment failure hospitalized for splenic aspirate were also included in the study. Demographic data were collected from all persons. Healthy persons from two villages in the VL-endemic region were recruited in February 2007 to provide control specimens. Participants included family members and neighbors of VL patients who had no signs or symptoms of VL and no history of VL.

Peripheral blood samples (400 μL) from all clinically suspect or treated patients and healthy controls were collected in Multivette 600 EDTA tubes (Sarstedt AG & Co., Nümbrecht, Germany). Spleen aspirates (100–200 μL) from seven clinically suspect patients and the three non-responding patients in 2005 and from 33 clinically suspect patients in 2007 were collected in tubes containing 200 μL of NET 10 buffer (10 mM NaCl, 10mM EDTA, 10 mM Tris-HCl, pH 8.0).

Serologic diagnosis. All specimens were evaluated by using four serologic tests performed by experienced professionals blinded to the participant's clinical status and other test results.

Fresh peripheral whole blood samples were tested at the AZHC by using two rK39 rapid tests (Kalazar Detect® Rapid Test; InBios International Inc., Seattle, WA, and DiaMed-IT Leish®; DiaMed AG, Cressier sur Morat, Switzerland) according to the manufacturers' instructions. The entire process takes approximately 10 minutes for the Kalazar Detect® test and 20 minutes for the DiaMed-IT Leish®. Whole blood or serum can be used for most rapid diagnostic tests. Although the manufacturer's instructions specify serum for the Kalazar Detect® test, there is now sufficient evidence that whole blood produces similar results for patients with active VL.[22]

The DAT (ITMA-DAT/VL; Institute of Tropical Medicine, Antwerp, Belgium) was performed by using freeze-dried antigen. The titration method followed the manufacturer's protocol; titers ≥ 1:3,200 were positive and DAT titers ≤ 1:400 were negative. In the case of high clinical suspicion of VL and a negative DAT result, the DAT was repeated after 4–5 days. If the DAT titer was in the range of 1:800–1:1,600, a splenic aspirate was performed.[21] Splenic aspirates were also performed for confirmation of VL in a group of suspected VL patients during the second recruitment period. The IFAT analysis on plasma samples was performed according to a standard method at the WHO Collaborating Center for Leishmaniasis in Madrid, Spain (WHO CC); the threshold titer for positivity was 1:80.[23] The performance of VL serologic tests is influenced by human immunodeficiency virus (HIV) co-infection.[23,24] However, routine HIV screening had not been instituted at the AZHC at the time of the study.

Parasitologic diagnosis. Spleen aspirate smears were stained with Giemsa stain. Two experienced microscopists independently examined each slide for at least one hour. Positive results were confirmed by examination at a magnification of 1,000×. One hundred microliters of spleen aspirate dilution were inoculated in Novy-MacNeal-Nicolle (NNN) medium for parasite isolation. Cultures were incubated at 27°C at the WHO CC (first two weeks at room temperature under field conditions) and examined under light microscopy every week for promastigote forms before subculturing with fresh medium. Subcultures were performed for four weeks before a negative result was provided.

A *Leishmania* nested PCR was performed to detect leishmanial DNA in peripheral blood and spleen aspirate samples at the WHO CC. One hundred microliters of peripheral blood and 100 μL of spleen aspirate dilution were subjected to a classical phenol-chloroform DNA extraction and ethanol precipitation, and 10 μL of the extracted DNA were analyzed by PCR according to the method of Cruz and others.[25,26] Standard quality control procedures were followed. In all assays, negative controls without DNA and controls with healthy human DNA were used. DNA from an estimated 10 promastigotes was used as the positive control in each experiment.[25,26]

Statistical analysis. Data were entered into an Excel (Microsoft, Redmond, WA) spreadsheet and statistical analysis was performed by using Epidat version 3.1 software (http://www.epidata.dk/). We first compared the performance of each serologic test to confirmed diagnosis based on positive results by PCR in peripheral blood and/or splenic aspirate. Because the sensitivity of PCR in peripheral blood was low, we also performed a separate analysis using data from all specimens tested by serology (DAT, IFAT and both rapid tests) and PCR

TABLE 1

Characteristics of participants recruited in 2005 and 2007 in Addis Zemen Health Center, Ethiopia*

Characteristic	Patient category		
	Suspected VL cases† (n = 125)	VL patients under treatment (n = 54)	Controls (n = 67)
Recruitment year			
2005, no. (%)	89 (70.6)	37 (68.5)	0 (0)
2007, no. (%)	36 (29.4)	17 (31.5)	67 (100)
Sex‡			
M, no. (%)	83 (71.6)	41 (75.9)	32 (48.5)
F, no. (%)	33 (28.4)	13 (24.1)	34 (51.5)
Age, years, mean (range)§	19.6 (2.4–60)	28.1 (11–60)	18.5 (3–53)

*VL = visceral leishmaniasis.
†Includes three patients who failed to respond clinically to antimonial treatment.
‡Sex data were missing for nine suspected patients with VL and one control.
§Age data were missing for nine suspected patients with VL and two controls.

TABLE 2

Comparative results of serologic and parasitologic tests and PCR for study population, Ethiopia*

Test	Patient category (no. positive/total)†			
	Suspect VL cases	Treatment failure‡	Under treatment§	Controls
Kalazar Detect	68/122	2/3	44/54	1/67
DiaMed-IT Leish	64/122	2/3	43/54	4/67
DAT	57/122	3/3	43/54	1/67
IFAT	72/122	3/3	47/54	1/67
PCR for peripheral blood	21/122	0/3	5/54	0/67
PCR for splenic aspirate	20/40	1/3	0/0	0/0
Culture for splenic aspirate	13/40	0/3	0/0	0/0
Microscopy for splenic aspirate	3/7	0/3	0/0	0/0

*VL = visceral leishmaniasis; DAT = direct agglutination test; IFAT = indirect immunofluorescent antibody test; PCR = polymerase chain reaction.
†No. specimens with positive results per total number of specimens tested.
‡VL patients with persistent clinical symptoms at the end of antimonial treatment.
§VL patients under treatment at the time of specimen collection.

in peripheral blood. Because none of the available tests for VL is considered to be a true gold standard, a latent class model was used. This model treated the true disease status as a latent (unobserved) variable assumed to determine the observed test results, and provided estimates of sensitivity and specificity for each test. An additional latent variable enabled conditional dependence between the InBios and DiaMed tests, which are based on the same rK39 antigen and are therefore expected to have correlated results.[27] Latent class analysis was performed using the software BLCM: Bayes Latent Class Models version 1.3.[28]

RESULTS

The study population included 246 participants recruited during the two study periods: 86 patients clinically suspected to have VL, 37 patients being treated for VL and 3 VL patients with clinically diagnosed treatment failure in October 2005, and 36 patients with suspect VL, 17 patients being treated for VL, and 67 healthy controls from a VL-endemic area in February 2007 (Table 1). Peripheral blood specimens from all participants were tested by PCR. Forty splenic aspirate specimens were obtained from suspect VL and treatment failure patients and tested by microscopy, culture, and PCR. A total of 35 patients (all of whom were suspected clinically to have VL) had positive PCR results: 12 in blood and splenic aspirate, 9 in splenic aspirate but not blood, and 14 in blood from patients who had no splenic aspirate obtained (Table 2). Thirteen PCR-positive splenic aspirate specimens were also positive by parasite culture and three of these by microscopy; no PCR-negative specimen was positive by classical parasitologic methods. Among the 179 patients with a clinical picture consistent with VL, 103 had a positive DAT titer (≥ 1:3,200). Three patients with negative or borderline DAT titers had positive results by PCR (two in blood, one in blood and splenic aspirate). All healthy controls from the VL-endemic area had negative results by blood PCR, but one person had a positive DAT titer.

In the analysis that considered the 35 specimens with positive PCR results to represent confirmed infection, the respective sensitivities were 94.3% for Kalazar Detect® test, 91.4% for DiaMed-IT Leish® and DAT, and 100% for IFAT; all tests showed 98.5% specificity except the DiaMed-IT Leish®, for which the specificity was 94% (Table 3). One healthy control had positive results for all serological tests and was considered to have a subclinical infection.

The latent class analysis was performed using diagnostic test data from all 246 specimens. Based on this analysis, the estimated sensitivities were 90.5%, 89%, 88.8%, and 96% for the Kalazar Detect® test, DiaMed-IT Leish® DAT, and IFAT, respectively (Table 4). Of the serologic tests, DAT showed the highest estimated specificity but the lowest sensitivity. Peripheral blood PCR had an estimated sensitivity of 23.2% and was highly specific.

DISCUSSION

In the Indian subcontinent, rK39 rapid diagnostic tests are now widely used for routine confirmation of VL in peripheral health care facilities and are considered important tools in the ongoing elimination effort in the region.[29] These tests have not been as widely accepted in VL-endemic settings in eastern Africa, in part because of reported lower sensitivity and specificity in published analyses.[11,12,16] However, our data suggest that in Ethiopia the sensitivity and specificity of the two most widely used rK39 rapid tests are at least as high as those of the DAT, and well within the acceptable range to be used as the first-line tool for confirmatory testing in peripheral health care facilities.

Our study had a number of limitations, most related to the logistical difficulties of conducting an epidemic investigation in a remote area. One of the most salient limitations was that only 35 of our VL patients had a proven molecular or parasitologic diagnosis. For this reason, we performed two analyses, one using suspect VL cases with positive PCR results as the comparator for sensitivity, and the second a latent class analysis that enabled us to include all specimen results. The sensitivity of the PCR assay was substantially lower than that seen in studies in Spain.[25,26] The low sensitivity was likely caused by the lack of optimal control over conditions between specimen collection in rural Ethiopia and their arrival at the laboratory in Madrid where molecular assays were conducted. We

TABLE 3

Comparative results of serologic tests for 35 VL patients with a diagnosis confirmed by PCR in peripheral blood and/or splenic aspirate and 67 healthy controls from the same VL-endemic area, Ethiopia*

Result	Kalazar Detect		Diamed-IT Leish		DAT		IFAT	
PCR	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Positive	33	2	32	3	32	3	35	0
Negative	1	66	4	63	1	66	1	66
Sensitivity (95% CI)	94.3 (85.2–100)		91.4 (80.7–100)		91.4 (80.7–100)		100 (98.6–100)	
Specificity (95% CI)	98.5 (94.9–100)		94.0 (87.6–100)		98.5 (94.9–100)		98.5 (94.9–100)	

*VL = visceral leishmaniasis; PCR = polymerase chain reaction; DAT = direct agglutination test; IFAT = indirect immunofluorescent antibody test; CI = confidence interval.

TABLE 4

Sensitivity and specificity for five VL diagnostic tests for 246 participants, Ethiopia*

Test	Sensitivity (%)		Specificity (%)	
	Estimate	95% Credible interval	Estimate	95% Credible interval
Kalazar Detect	90.5	84.0–95.2	90.7	84.3–95.5
DiaMed-IT Leish	89.0	81.7–94.3	91.0	85.1–95.3
DAT	88.8	81.5–94.3	97.4	93.3–99.4
IFAT	96.0	90.8–98.8	89.6	83.1–94.5
PCR for peripheral blood	23.2	16.3–31.4	99.5	97.2–100

*VL = visceral leishmaniasis; DAT = direct agglutination test; IFAT = indirect immunofluorescent antibody test; PCR = polymerase chain reaction. Estimates and credible intervals are based on Bayesian latent class analysis assuming pairwise correlation between InBios and DiaMed rK39 rapid tests.

included 54 VL patients under treatment at the time of sampling; not surprisingly, more than 90% were PCR negative. However, we believe that serologic results were still likely to be reliable because reversion to negative serologic results is slow in treated VL patients, taking more than a year in most cases.[30]

Unfortunately, our study was conducted at a time when routine HIV testing was not available at the field site. Subsequent routine HIV testing demonstrated that 18% of adult VL patients treated at the AZHC in 2007 had co-infection with HIV; the prevalence in the general population was estimated to be 2.1%.[31] In data from Europe, HIV–VL co-infected patients had low and even negative antibody titers against *Leishmania*.[23] A prior study in Ethiopia demonstrated that the DAT remained reasonably sensitive (89%) whereas the sensitivity of DiaMed-IT Leish® was 77% in specimens from HIV–VL co-infected patients.[24] Nevertheless, because the presence of a substantial number of undiagnosed HIV-co-infected patients in our study would lead to an underestimate of test sensitivity, our current estimates may in that case represent a lower bound for HIV-negative patients. Fortunately, HIV screening is now much more widely available for VL patients in Ethiopia, which enables a more informed interpretation of rapid test results for these patients.

The results of our analysis differ from earlier reports. The meta-analysis published in 2006 estimated the overall sensitivity and specificity of the rK39 ICTs at 93.9% (95% confidence interval [CI] = 87.7–97.1%) and 95.3% (95% CI = 88.8–98.1%), respectively. However, in studies in eastern Africa, the estimated sensitivity and specificity were only 79% (95% CI = 46.7–94.2%) and 85.2% (95% CI = 28.2–98.8%).[11] In both of our analyses, the two rK39 ICTs had equivalent sensitivity to DAT with specificities above 90%. Estimates of ICT performance in eastern Africa combine data across a region known for genetic heterogeneity of *L. donovani* (in marked contrast to the Indian sub-continent where the parasites that cause VL are remarkably homogeneous and rK39 rapid test performance is consistently excellent).[11,12,32,33] The performance of serologic tests may vary depending on the characteristics of the circulating parasites, differences in the populations tested, and the rapid test formulations. The ability to make most VL diagnoses without the need for specialized equipment or invasive procedures makes these tests a major advance for peripheral health care facilities. We believe that our data form the basis for recommending the use of an rK39 ICT as the first-line test to confirm VL in patients meeting the appropriate case definition in rural Ethiopia.

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